PROPERTIES OF CULTURES OF ANTHRAX BACILLI GROWN AT VARIOUS TEMPERATURES

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PROPERTIES OF CULTURES OF ANTHRAX BACILLI GROWN AT VARIOUS TEMPERATURES

[Following is the translation of an article by I. F. Batyuk, Department of Microbiology (Head -- Prof. S. S. Dyachenko) of the Kiev Order of the Labor Red Banner of the Medical Institute imeni Academy Member A. A. Bogomolets (Director -- Associate Prof. I. P. Alekseyenko), published in the Russian-language periodical J. of Microbiology, No. 1, 1956, pp 19-22. It was submitted on 23 May 1955. Translation performed by Sp/7 Charles T. Ostertag Jr.]

The study of the conditions which determine the trend of regular mutability of microorganisms has a great theoretical and practical significance.

The great Russian scientist I. I. Mechnikov already initiated the development of the materialistic trend of the theory of development of microbes. Then in the beginning of the 1890's, L. Pasteur and L. S. Tsenkovskiy, with a number of brilliant experiments, showed that changing the temperature conditions for the incubation of anthrax cultures could weaken their virulence. As a result of cultivation at 42-43° they were able to obtain qualitatively new anthrax cultures -- less virulent, but retaining the immunogenic properties of the old species. Tsenkovskiy demonstrated the stability of the biological and immunogenic properties of the strains obtained by him, which were used with success for the preparation of live anthrax vaccine. In doing this work, Pasteur and Tsenkovskiy showed that it was possible to weaken the virulence of the anthrax causative agent only in the event that during cultivation they were deprived of the capability for spore formation. For this the seedings of a vegetative form of culture (blood from an anthrax patient) were made in broth and incubated, as pointed out above, at 42-43°.

Modern data makes it possible to confirm that for obtaining a persistent immunity against anthrax it is necessary to administer a live weakly virulent culture to an animal, since the organism reacts specifically, less intensively, to live anthrax vaccines than to the unchanged causative agent of the disease, and together with this acquires an immunity to anthrax.

In the present article we have undertaken the mission to make a more thorough study of the effect of high temperatures on the virulence, immunogenic and biochemical properties of the anthrax bacillus and substantiate a method for obtaining more effective and less virulent vaccine strains.

In the tests we used three strains of an anthrax culture which were being studied in our laboratory and which were virulent in the vegetative form -- No. 1909, Barsuk and Kon No. 3. Rabbits were infected with the culture for the purpose of obtaining the vegetative form. The culture was administered subcutaneously in the amount of 0.2 ml. The rabbits usually died in 59-63 hours. The dead rabbits were dissected and the blood, taken aseptically from the heart, was inoculated in flasks with broth, prepared according to Tsenkovskiy's prescription. The seedings of blood were placed in an incubator at a temperature of 43° and 45°.

In the first test we studied the influence of various periods of incubation at 43° on the vegetative form of the culture. Beginning with the fourth day following seeding, daily we removed one flask from the incubator, and from it made a screening on agar, which was placed at a temperature of 35° for 4--5 days for spore formation. The pure spore form of the culture was suspended in 40% glycerin.

The virulent properties of the cultures obtained were tested on guinea pigs and rabbits. In the tests we used 27 pigs and 8 rabbits which received intracutaneously the culture with the various incubation periods. The guinea pigs, weighing 400 to 600 g received 0.2 ml each, and the rabbits, weighing 1800-2000 g -- 0.5 ml of the culture each. The results of the test showed that a culture of the anthrax causative agent, which was incubated at 43° for 35 days, turned out to be virulent and caused the death of all the experimental animals. Thus we did not obtain the same results which were described in the tests of Tsenkovskiy and Pasteur.

It was established in subsequent special investigations that at 43° the vegetative form of a culture of the anthrax causative agent may transform into the spore form.

In the second test we used one of the virulent cultures of the anthrax causative agent for the investigation. With it we infected two rabbits. The animals died in the minimal periods. The blood was taken from the heart of the dead animal with the capillary of a thin pipette and sown on the bottom of test tubes with broth. Without being shaken up, the seedings were transferred to an incubator for incubation at 45°. Every 24 hours one test tube was taken from the incubator, seedings were made from it on agar for spore formation, and the resulting spore form of the culture was preserved in 40% glycerin.

The virulence of cultures from various periods of incubation was studied on white mice, guinea pigs and rabbits. The guinea pigs, weighing from 300 to 500 g received 0.2 ml intracutaneously, and the rabbits,

weighing from 1200--2500 -- 0.5 ml of the culture each. The white mice were infected under the skin in doses of 0.01 and 0.1 ml.

As is seen from table 1, the culture incubated at 45° for six days killed all the white mice and guinea pigs and from 30--50% of the rabbits. The culture, obtained following incubation under the same conditions for seven days, turned out to be less virulent -- it killed all the white mice, from 20--30% of the guinea pigs, and did not kill the rabbits.

In this manner, in the second test we were able to change the virulence of the investigated strain, and after 6 and 7 days to obtain cultures which in virulence were close to the Tsenkovskiy vaccine. This gave us the basis to consider that at a temperature of 45° the bacilli of anthrax did not form spores, since in the spore form of the culture at the same incubation temperature, no noticeable changes in virulence set in.

It could be thought that under the stated conditions of incubation, there took place not only a weakening of virulence, but also changes of other properties -- biochemical activity, antigenic structure, etc. Therefore it was necessary to more thoroughly study the properties of the cultures obtained. We were particularly interested in the culture of the 7 day incubation, which in virulence stood between the first and second Tsenkovskiy vaccine.

Its cultural properties were studied on meat-peptone agar and in meat-peptone broth (pH = 7.2--7.4). On the agar the culture grew in the form of irregular, fringed formations, interwoven by chains. Individual colonies were small, flat, of an erratic form and gray color. In the broth the culture grew in the form of a small, little noticeable, blur with a precipitate of slightly yellowish color. Upon shaking the precipitate broke into a homogeneous suspension.

Upon microscopic investigation of 24-hour seedings in a crushed drop and stained preparations, the culture presented the bacillary form of the anthrax causative agent, in the shape of immobile, uniform, gram-positive bacilli with a length of 6--9 microns and a thickness of 1--2 microns. Morphologically changed forms were few.

The study of the biochemical properties of the culture was carried out on media with lactose, glucose, mannitol, maltose, saccharose, and also in Hottinger broth (for determining indole formation) and milk (for determining peptonizing properties). The culture decomposed glucose and maltose with the formation of acid without gas. It decomposed mannitol and saccharose inconsistantly and curdled, and then peptonized, milk. It did not form indole and formed traces of H_2S.

As can be seen from the data presented, based on morphological, cultural and biochemical properties, in the culture obtained by us there were no deviations detected from the properties of the initial culture.

We set up two tests for the purpose of studying the vaccinating properties of the culture.

The first test was conducted on 30 rabbits and 18 guinea pigs, to whom the culture was administered twice with an interval of seven days, intracutaneously to one group and subcutaneously to the second in an amount of 0.2 ml for guinea pigs and 0.5 ml for rabbits. There were 55-60 million spores in 1 ml of this culture.

In the second test the culture was administered to 20 rabbits and 7 guinea pigs one time, subcutaneously and intracutaneously in the same volume of doses but the concentration of spores in 1 ml of culture was greater -- 75--80 million. In 31 days the vaccinated animals of the first test, and in 25 days the vaccinated animals of the second test were infected with a lethal dose of a virulent culture of the anthrax causative agent which was titrated for guinea pigs and rabbits.

In analyzing the results presented in table 2, it can be said that a culture of a 7-day incubation in chicken broth (pH = 7.1 --7.2) at 45° possessed immunogenic properties. Vaccinated laboratory animals, subsequently infected with a virulent culture of the anthrax causative agent, remained alive in the majority of cases. Control animals died over the expanse of three days. All the dead animals were subjected to a bacteriological investigation, and in all cases a culture of the anthrax microbe was isolated.

Following the intracutaneous vaccination of animals, the 7-day culture did not cause a strong reaction and large edemas at the site of administration, and as a result of a single inoculation an intense immunity was created.

The high immunogenicity of the investigated culture (apart from the conditions of cultivation) should probably be explained also by the fact that the culture preserved the property of capsule formation.

The cited tests gave us the basis to conclude that incubation at 45° may be utilized with the aim of weakening the virulence of anthrax cultures.

Conclusions

1. When incubating a culture of anthrax bacilli at 43° we observed the formation of spores in them. Therefore, in spite of the generally acknowledged opinion, established in the literature since the time of

Tsenkovskiy, we cannot consider incubation of anthrax cultures at the stated temperature as a completely satisfactory method for obtaining anthrax strains.

2. By incubating a culture of anthrax bacilli in meat-peptone chicken broth with a pH = 7.1 - 7.2 at 45°, we established that under these conditions they do not transform from the vegetative form into the spore form. During this the morphological, cultural and biochemical properties of the culture did not change.

3. In a culture obtained following incubation of a virulent strain of the anthrax causative agent in broth at 45° for 7 days, a low virulence was combined with high immunizing properties -- after a single inoculation an intense immunity set-in in the animals.

Table 1

Period of incubation in days	Results of tests					
	White mice		Guinea pigs		Rabbits	
	Died	Lived	Died	Lived	Died	Lived
1--5	All died after 48--70 hours	-	100%	-	100%	-
6	Same	-	100%	-	30-50%	50-70%
7	Same	-	20-30%	70-80%	-	100%
8--9	Same	-	-	-	-	100%

Table 2

No. of test	Method of vaccination	Guinea pigs			Rabbits		
		Total	Lived	Died	Total	Lived	Died
1	Double	13	8	5	30	21	9
2	Single	7	4	3	20	11	6
	Nonvaccinated animals Control	3	-	3	2	-	2